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Characteristic defects in neural crest cell-specific $G\alpha_q/G\alpha_{11}$- and $G\alpha_{12}/G\alpha_{13}$-deficient mice

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Abstract

The endothelin/endothelin receptor system plays a critical role in the differentiation and terminal migration of particular neural crest cell subpopulations. Targeted deletion of the G-protein-coupled endothelin receptors ET_A and ET_B was shown to result in characteristic developmental defects of derivatives of cephalic and cardiac neural crest and of neural crest-derived melanocytes and enteric neurons, respectively. Since both endothelin receptors are coupled to G-proteins of the G_q/G_{11}- and G_{12}/G_{13}-families, we generated mouse lines lacking $G\alpha_q/G\alpha_{11}$ or $G\alpha_{12}/G\alpha_{13}$ in neural crest cells to study their roles in neural crest development. Mice lacking $G\alpha_q/G\alpha_{11}$ in a neural crest cell-specific manner had craniofacial defects similar to those observed in mice lacking the ET_A receptor or endothelin-1 (ET-1). However, in contrast to ET-1/ET_A mutant animals, cardiac outflow tract morphology was intact. Surprisingly, neither $G\alpha_q/G\alpha_{11}$- nor $G\alpha_{12}/G\alpha_{13}$-deficient mice showed developmental defects seen in animals lacking either the ET_B receptor or its ligand endothelin-3 (ET-3). Interestingly, $G\alpha_{12}/G\alpha_{13}$ deficiency in neural crest cell-derived cardiac cells resulted in characteristic cardiac malformations. Our data show that G_q/G_{11}- but not G_{12}/G_{13}-mediated signaling processes mediate ET-1/ET_A-dependent development of the cephalic neural crest. In contrast, ET-3/ET_B-mediated development of neural crest-derived melanocytes and enteric neurons appears to involve G-proteins different from $G_q/G_{11}/G_{12}/G_{13}$.

Keywords: G-proteins; Neural crest; Endothelin; Craniofacial development; Cardiovascular development; Knock-out; Mouse

Introduction

The neural crest is a population of pluripotent cells, which originates from the dorsal part of the neural tube. After delamination from the neural tube, neural crest cells migrate to the periphery via characteristic paths. Once they have reached particular sites, they stop to move and differentiate into a wide variety of different cell types. These include cells of the peripheral nervous system, enteric ganglia, endocrine chromaffin cells of the adrenal medulla, melanocytes, most cells of the craniofacial skeletal and connective tissue as well as part of the heart outflow tract (Le Douarin and Kalcheim, 1999). The loss of pluripotency of neural crest cells and their gradual differentiation to particular cell types requires a complex interplay of cell-autonomous processes and the action of environmental signals.

Among the receptors on migrating neural crest cells which receive signals from environmental cues are the G-protein-coupled endothelin ET_A and ET_B receptors (Kurihara et al., 1999). Genetic studies have demonstrated that mice lacking ET_A or its ligand endothelin-1 (ET-1) show defects in the development of derivatives of cephalic and cardiac neural crest cells including branchial arch-derived craniofacial tissue and the cardiac outflow tract (Clouthier et al., 1998; Kurihara et al., 1994, 1995). Mice lacking ET_B or its ligand endothelin-3 (ET-3) have alterations in coat color and develop an aganglionic megacolon due to defects in the development of neural crest-derived melanocytes and enteric

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neurons (Baynash et al., 1994; Hosoda et al., 1994). Both the ET-1/ET_A and the ET-3/ET_B signaling pathways are involved in the correct differentiation and/or terminal migration of particular neural crest cell subpopulations (Barlow et al., 2003; Clouthier et al., 2000; Lee et al., 2003). Signaling via ET_A and ET_B receptors during neural crest cell development occurs in a cell-autonomous and non-autonomous fashion (Clouthier et al., 2003; Hou et al., 2004).

Activated ET_A receptors signal through the G-proteins G_q/G_{11} and G_{12}/G_{13} resulting in the activation of β-isoforms of phospholipase C and the activation of RhoA-mediated signaling, respectively (Gohla et al., 1999, 2000; Kedzierski and Yanagisawa, 2001; Oksche, 2004). Signals from ET_B receptors are transmitted by G_i/G_o, G_q/G_{11}, as well as G_{13} (Kedzierski and Yanagisawa, 2001; Kitamura et al., 1999; Oksche, 2004). Mice lacking both $G\alpha_q/G\alpha_{11}$ or $G\alpha_{12}/G\alpha_{13}$ die at e9.5 or earlier due to developmental defects of the cardiovascular system (Gu et al., 2002; Offermanns et al., 1997, 1998). Although this early embryonic lethality does not allow to study the role of G_q/G_{11} and G_{12}/G_{13} in neural crest cell development, the analysis of e9.5 $G\alpha_q^{-/-};G\alpha_{11}^{-/-}$ animals and of $G\alpha_q^{-/-};G\alpha_{11}^{-/+}$ neonates has provided some evidence that G_q/G_{11} are involved in ET-1/ET_A signaling in the pharyngeal arch mesenchyme (Ivey et al., 2003; Offermanns et al., 1998).

In order to study the role of G_q/G_{11}- and G_{12}/G_{13}-mediated signaling in neural crest cell development, we have generated conditional mouse mutants lacking $G\alpha_q/G\alpha_{11}$ or $G\alpha_{12}/G\alpha_{13}$ selectively in migrating neural crest cells and their derivatives.

Materials and methods

Transgenic and mutant mice

P0-Cre transgenic mice (Yamauchi et al., 1999) were kindly provided by Dr. Yuji Mishina (NIH, Research Triangle Park, NC, USA). $G\alpha_q^{flox/flox};G\alpha_{11}^{-/-}$ as well as $G\alpha_{13}^{flox/flox};G\alpha_{12}^{-/-}$ animals were generated as previously described (Gu et al., 2002; Ivey et al., 2003; Moers et al., 2003; Offermanns et al., 1998; Wettschureck et al., 2001). Rosa26lacZ mice (Soriano, 1999) were obtained from The Jackson Laboratory (Bar Harbor, ME, USA).

Genotyping

Genotypes of the embryos and adult animals were determined by polymerase chain reaction (PCR). Genomic DNA was isolated from yolk sac or tail biopsies of live embryos, fetuses, newborns, and postnatal day 21 mice. Primers used for detecting P0-Cre gene were 5′-ATTGGTCACTGGCTCAGGACAG-3′ and 5′-GCATAACCAGTGAAACAGCATTGC-3′. Primers used for detecting the floxed *gna*q allele were 5′-AGCTTAGTCTGGTGACAGAAGC-3′ and 5′-GCATGCGTGTCCTTTATGTGAG-3′. Primers used for detecting floxed *gna*13 allele were 5′-GCACTCTTACAGACTCCCAC-3′ and 5′-GCCACAGAGGGATTCAGCAC-3′. Primers used for detecting wildtype and *gna*11 alleles were 5′-CAGGGGTAGGTGATGATTGTGC-3′ and 5′-AGCATGCTGTAAGACCGTAG-3′ for wildtype and 5′-GACTAGTGAGACGTGCTACTTCC-3′ for null allele. Primers used for detecting *gna*12 gene were 5′-GTGCTCATCCTTCCTGGTTTCC-3′ and 5′-CGGGTCGCCCTTGAAATCTGG-3′ for wildtype and 5′-GGCTGCTAAAGCGCATGCTCC-3′ for null allele. To genotype Rosa26lacZ transgenic animals, three oligonucleotides were used, as previously reported (Soriano, 1999).

Detection of β-galactosidase activity

e14.5 and e18.5 mouse embryos expressing lacZ transgenes were harvested, fixed overnight at 4°C in 0.2% paraformaldehyde solution followed by overnight incubation at 4°C in 30% sucrose in phosphate buffer. Embryos were then frozen in OCT on dry ice. Frozen embryos were cryosectioned at 20 μm thickness and mounted on gelatine-coated slides. Sections were stained overnight at 37°C in X-gal staining solution and counterstained with eosin. 6 and 12 animals were analyzed at e18.5 and e14.5, respectively.

General histology and skeletal preparation

Embryos were harvested at e18.5 and fixed overnight in 10% neutral buffered formalin and processed for histology. Paraffin sections were cut at 10 μm thickness and stained with hematoxylin and eosin according to routine histological procedures. For skeletal preparations, skin and viscera were removed from freshly harvested embryos, followed by alizarin red S (Sigma, Deisenhofen, Germany) and alcian blue 8GX (Sigma, Deisenhofen, Germany) staining as described (Kaufman, 1992). At least 12 animals were analyzed per genotype.

Whole-mount in situ hybridization

Whole-mount in situ hybridization was performed as described (Henrique et al., 1995) using digoxigenin-labeled antisense riboprobes synthesized from cDNAs. *CrapbI* and *Hoxa2* cDNAs were kindly provided by Dr. Moises Mallo (Instituto Gulbenkian de Ciencias, Oeiras, Portugal), cDNAs for *eHAND* and *dHAND* were kindly provided by Dr. Eric N. Olson (Department of Molecular Biology, Southwestern Medical School, Dallas, TX, USA), *Dlx2* and *Dlx5* cDNAs were kindly provided by Dr. John L. Rubenstein (Department of Psychiatry, Nina Ireland Laboratory of Developmental Neurobiology, San Francisco, CA, USA) and *Dlx3* cDNA were kindly provided by Prof. Kenneth M. Weiss (Department of Anthropology, Univer-

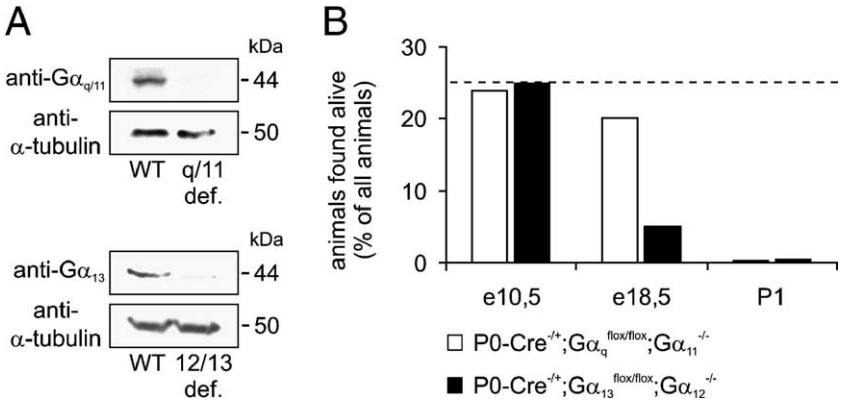

Fig. 1. (A) Western blot of lysates prepared from dorsal root ganglia of e18.5 wildtype (WT), P0-Cre$^{-/+}$;Gα$_q^{flox/flox}$;Gα$_{11}^{-/-}$ (q/11 def.; upper panel) and P0-Cre$^{-/+}$;Gα$_{13}^{flox/flox}$;Gα$_{12}^{-/-}$ mice (12/13 def.; lower panel) using anti-Gα$_{q/11}$ and anti-Gα$_{13}$ antibodies. Immunoblots developed with anti-α-tubulin antibody were performed as controls. (B) Survival rate of neural crest cell-specific Gα$_q$/Gα$_{11}$-deficient and Gα$_{12}$/Gα$_{13}$-deficient mice during development. Shown are the percent of P0-Cre$^{-/+}$;Gαq$^{flox/flox}$;Gα$_{11}^{-/-}$ and P0-Cre$^{-/+}$;Gα$_{13}^{flox/flox}$;Gα$_{12}^{-/-}$ animals found alive in offspring of P0-Cre$^{-/+}$;Gα$_q^{+/flox}$;Gα$_{11}^{-/-}$ × Gα$_q^{flox/flox}$;Gα$_{11}^{-/-}$ or P0-Cre$^{-/+}$;Gα$_{13}^{+/flox}$;Gα$_{12}^{-/-}$ × Gα$_{13}^{flox/flox}$;Gα$_{12}^{-/-}$ matings, at the indicated developmental stage. White columns represent Gα$_q$/Gα$_{11}$-deficient mice; black columns represent Gα$_{12}$/Gα$_{13}$-deficient animals. Hatched horizontal line indicates the expected percentage level of 25. Total numbers of animals evaluated were: 67 (e10.5); 322 (e18.5); 385 (P1).

sity Park, PA, USA). At least 6 embryos were analyzed per probe and genotype.

Immunofluorescence and Western blotting

For immunofluorescent staining, 10 μm paraffin sections from e18.5 embryos were incubated with monoclonal anti-α smooth muscle actin antibody (1:400; Sigma, Deisenhofen, Germany) overnight at 4°C, followed by washing and incubation with the FITC-conjugated goat anti-mouse antibody (1:200, Dianova, Hamburg, Germany) at room temperature for 30 min. After washing, stained cells were visualized using a fluorescence microscope (Leica). 7 animals were analyzed per genotype. Western blotting was

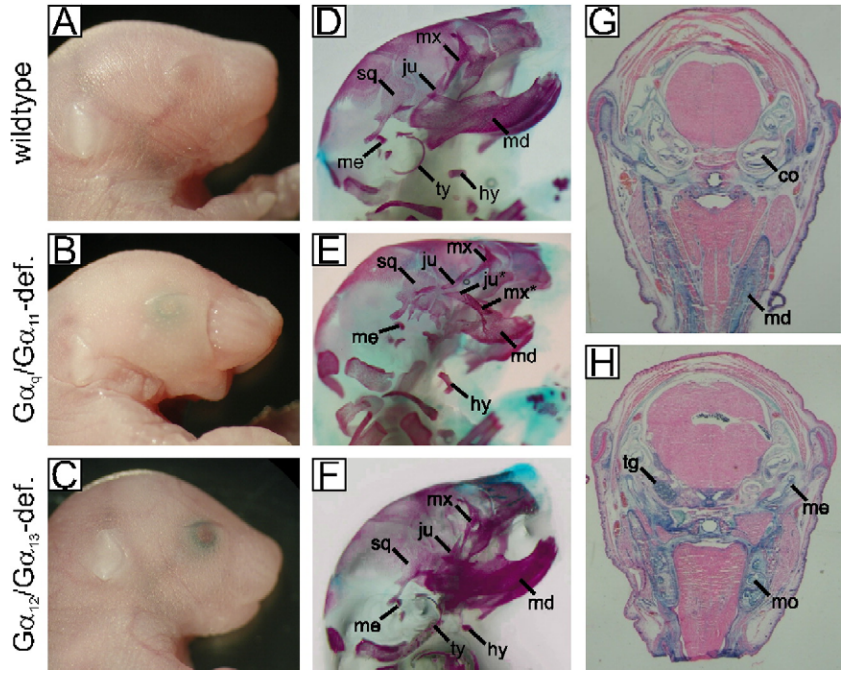

Fig. 2. Craniofacial phenotype in neural crest cell-specific Gα$_q$/Gα$_{11}$-deficient and Gα$_{12}$/Gα$_{13}$-deficient mice. (A–C) Head morphology of e18.5 embryos of wildtype (A), Gα$_q$/Gα$_{11}$-deficient (B) and Gα$_{12}$/Gα$_{13}$-deficient (C) mice. (D–F) Lateral views of skull preparations of e18.5 wildtype (D), Gα$_q$/Gα$_{11}$- (E) and Gα$_{12}$/Gα$_{13}$-deficient embryos (F) stained with alizarin red (bone) and alcian blue (cartilage). (G, H) P0-Cre-mediated recombination pattern in the head of an e18.5 Rosa26lacZ$^{-/+}$;P0-Cre$^{-/+}$ mouse embryo, using β-galactosidase staining of transversal cryosections. sq—squamosal bone; md—mandible; ty—tympanic ring; ju—jugular bone; ju*—pseudo jugular bone; mx—maxilla; mx*—pseudo maxilla; hy—hyoid bone; co—cochlea; me—middle ear bone primordia; tg—trigeminal ganglion.

Table 1
Craniofacial and cardiac defects in e18.5 P0-Cre$^{-/+}$;G$\alpha_q^{flox/flox}$;G$\alpha_{11}^{-/-}$ and P0-Cre$^{-/+}$;G$\alpha_{13}^{flox/flox}$;G$\alpha_{12}^{-/-}$ mice, respectively

P0-Cre$^{-/+}$;G$\alpha_q^{flox/flox}$;G$\alpha_{11}^{-/-}$	
Shortened lower jaw	46/46 (100%)
Facial soft tissue defects	29/46 (63%)
Absent tympanic ring	5/11 (45%)
Duplicated maxilla	9/11 (82%)
Duplicated jugular bone	9/11 (82%)
P0-Cre$^{-/+}$;G$\alpha_{13}^{flox/flox}$;G$\alpha_{12}^{-/-}$	
Upper interventricular septum defect	10/12 (83%)
Overriding aorta	10/12 (83%)
Interventricular septum cavity	12/12 (100%)

performed according to standard procedures using anti-Gα_q/Gα_{11} (1:500) and anti-Gα_{13} (1:500) antibodies (Santa Cruz Biotechnology Inc., Heidelberg, Germany) or anti-α-tubulin antibody (Sigma, Deisenhofen, Germany).

Results

Since mice lacking Gα_q/Gα_{11} or Gα_{12}/Gα_{13} die at e9.5 or earlier (Gu et al., 2002; Offermanns et al., 1997, 1998), we employed conditional mutagenesis using the Cre/loxP system to analyze the role of these G-proteins in later stages of development. Since Gα_{11}- and Gα_{12}-deficient animals have no obvious phenotypic abnormalities (Gu et al., 2002; Offermanns et al., 1998), conditional inactivation of the Gα_q/Gα_{11}- as well as of the Gα_{12}/Gα_{13}-mediated signaling pathway can be performed by Cre-mediated recombination of the floxed Gα_q and Gα_{13} genes in Gα_{11}- or Gα_{12}-deficient mice, respectively. We could recently show that Cre-mediated in vivo deletion of Gα_q/Gα_{11} and Gα_{12}/Gα_{13} can be achieved with high efficiency (Moers et al., 2003; Wettschureck et al., 2001). To test the effect of Gα_q/Gα_{11} and Gα_{12}/Gα_{13} deficiency on the development of neural crest-derived structures, we have crossed

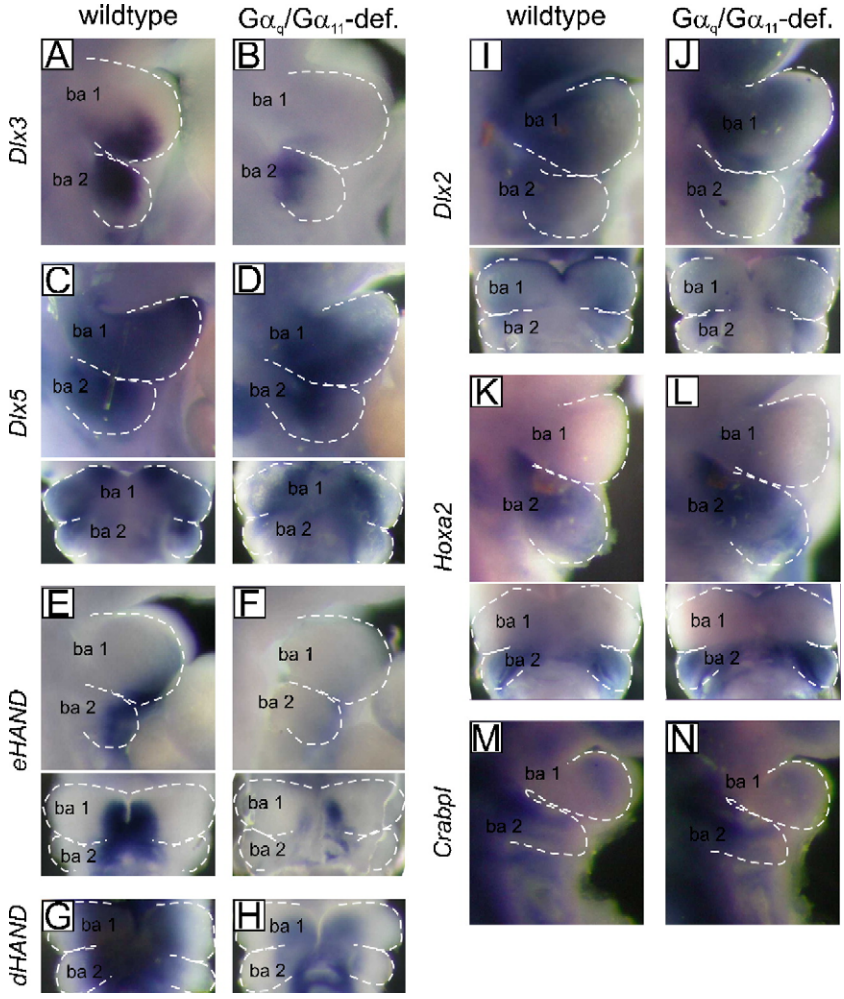

Fig. 3. Gene expression analysis in branchial arches of wildtype (A,C,E,G,I,K,M) and neural crest cell-specific Gα_q/Gα_{11}-deficient embryos (B,D,F,H,J,L,N). Shown are whole-mount in situ hybridizations using digoxigenin-labeled riboprobes specific for *Dlx3* (A,B), *Dlx5* (C,D), *eHAND* (E,F), *dHAND* (G,H), *Dlx2* (I,J), *Hoxa2* (K,L), and *CrabpI* (M,N). Embryos were prepared at e10.5 except for staining with *CrabpI* probe, which was performed on e9.5 embryos. (A–H) Downregulation of *Dlx3*, *Dlx5*, *eHAND*, and *dHAND* in neural crest cell-specific Gα_q/Gα_{11}-deficient embryos. (I–N) *Dlx2*, *Hoxa2*, and *CrabpI* expression is not altered in branchial arches of neural crest cell-specific Gα_q/Gα_{11}-deficient embryos. Please, note that similar to ET_A receptor-deficient mice (Ruest et al., 2004) epithelial *Dlx2* expression was lost in the Gα_q/Gα_{11} mutants. ba 1—mandibular prominence of the first branchial arch; ba 2—second branchial arch.

$G\alpha_q^{flox/flox};G\alpha_{11}^{-/-}$ as well as $G\alpha_{13}^{flox/flox};G\alpha_{12}^{-/-}$ animals with a mouse line expressing Cre under the control of the P0-promoter, which results in the recombination in tissues derived from neural crest cells such as the sympathetic nervous system, dorsal root ganglia, the enteric nervous system, the mesenchyme of the heart outflow tract and the craniofacial mesenchyme at stages later than e9 (Abu-Issa et al., 2002; Brennan et al., 2003; Crone et al., 2003; Hasegawa et al., 2002; Yamauchi et al., 1999). Fig. 1A demonstrates the absence of $G\alpha_q/G\alpha_{11}$ and $G\alpha_{13}$ in dorsal root ganglia of $P0\text{-}Cre^{-/+};G\alpha_q^{flox/flox};G\alpha_{11}^{-/-}$ and $P0\text{-}Cre^{-/+};G\alpha_{13}^{flox/flox};G\alpha_{12}^{-/-}$ mice. As shown in Fig. 1B, no alive $P0\text{-}Cre^{-/+};G\alpha_q^{flox/flox};G\alpha_{11}^{-/-}$ or $P0\text{-}Cre^{-/+};G\alpha_{13}^{flox/flox};G\alpha_{12}^{-/-}$ mice were found in the offspring of $P0\text{-}Cre^{-/+};G\alpha_q^{+/flox};G\alpha_{11}^{-/-} \times G\alpha_q^{flox/flox};G\alpha_{11}^{-/-}$ or $P0\text{-}Cre^{-/+};G\alpha_{13}^{+/flox};G\alpha_{12}^{-/-} \times G\alpha_{13}^{flox/flox};G\alpha_{12}^{-/-}$ matings at postnatal day 1. Genotyping of dead newborns identified neural crest cell-specific $G\alpha_q/G\alpha_{11}$-deficient as well as $G\alpha_{12}/G\alpha_{13}$-deficient animals indicating that they died shortly after birth. Close observation of newly born $G\alpha_q/G\alpha_{11}$ or $G\alpha_{12}/G\alpha_{13}$ mutant mice showed that $G\alpha_q/G\alpha_{11}$ mutant mice died within minutes after birth whereas $G\alpha_{12}/G\alpha_{13}$ mutant animals survived for several hours. When genotyping was carried out on pups born by Cesarean section at e18.5, only slightly reduced numbers of $G\alpha_q/G\alpha_{11}$ mutant animals were obtained, while the number of $G\alpha_{12}/G\alpha_{13}$ mutant pups was already reduced by about 80%. Expected numbers of $G\alpha_q/G\alpha_{11}$ and $G\alpha_{12}/G\alpha_{13}$ mutant embryos were found at e10.5. While $G\alpha_{12}/G\alpha_{13}$ mutant mice at e18.5 looked grossly normal, $G\alpha_q/G\alpha_{11}$ mutants showed severe craniofacial defects. As shown in Figs. 2A–C, the frontonasal region was malformed and the lower jaw was hypoplastic. Staining of the skeletal system of newborns with alizarin red and alcian blue revealed poorly developed mandibles, often absence of tympanic rings (Table 1). In addition, jugular and maxillar bones were duplicated resembling the phenotype found in ET-1- and ET_A receptor-deficient mice (Ozeki et al., 2004; Ruest et al., 2004) (Figs. 2D–F). The remaining bones of the facial skull appeared to be not affected in the mutants.

Cre-mediated recombination of the cranial neural crest can be seen in P0-Cre animals starting from e9.5 when the frontonasal mass, trigeminal ganglia, and branchial arches show Cre expression (Hasegawa et al., 2002; Yamauchi et al., 1999). The branchial arch 1 later develops into the maxillary and mandibular prominences which are the primordia of upper and lower jaws, respectively, as well as some of the middle ear bones as can be seen in e18.5 P0-Cre mice carrying the Rosa26lacZ reporter gene (Figs. 2G,H).

The transcription factors *Dlx3*, *Dlx5*, *dHAND*, and *eHAND* are expressed in the developing pharyngeal arches and are involved in craniofacial patterning (Merlo et al., 2000; Ruest et al., 2003; Yanagisawa et al., 2003). Similar to the observation made at e9.5/e10.5 ET_A receptor- and ET-1-deficient embryos (Charite et al., 2001; Clouthier et al., 2000; Ozeki et al., 2004; Ruest et al., 2004; Thomas et al., 1998), *Dlx3*, *eHAND*, and *dHAND* were downregulated in the first and second branchial arch of e10.5 neural crest cell-specific $G\alpha_q/G\alpha_{11}$-deficient mice (Figs. 3A,B,E–H), and *Dlx5* expression was reduced in the distal regions of branchial arches 1 and 2 (Figs. 3C,D). We then performed whole mount in situ hybridization to study the expression of several factors known to be expressed by migrating neural crest cells. The expression of *Dlx2*, *Hoxa2*, and *CrabpI* in branchial arches of neural crest cell-specific

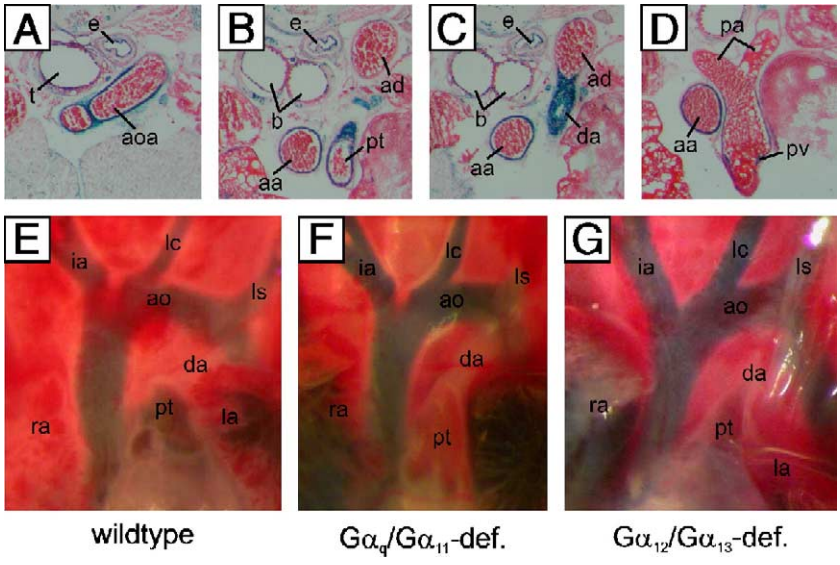

Fig. 4. Outflow tract morphology of the neural crest cell-specific $G\alpha_q/G\alpha_{11}$-deficient and $G\alpha_{12}/G\alpha_{13}$-deficient mice. (A–D) β-galactosidase staining of transversal cryosections of e18.5 $Rosa26lacZ^{-/+};P0\text{-}Cre^{-/+}$ embryos. (E–G) Morphology of the cardiac outflow tract of e18.5 embryonic hearts. Shown are the cardiac outflow tract structures filled with Evans blue of wildtype (E), $G\alpha_q/G\alpha_{11}$-deficient (F) as well as $G\alpha_{12}/G\alpha_{13}$-deficient mice (G). o—oesophagus; t—trachea; aoa—aortic arch; ad—aorta descendens; b—bronchi; aa—aorta ascendens; pt—pulmonary trunk; da—ductus arteriosus; pa—pulmonary arteries; pv—pulmonary valve; ia—innominate artery; lc—left common carotid artery; ls—left subclavian artery; ao—aorta; ra, la—right, left atrium, respectively.

$G\alpha_q/G\alpha_{11}$-deficient e9.5/e10.5 embryos was indistinguishable from the expression in wildtype embryos indicating that the expression of these genes was unaffected by $G\alpha_q/G\alpha_{11}$ deficiency (Figs. 3I–N). This is consistent with findings made in ET_A receptor- and in ET1-deficient mice (Clouthier et al., 2000; Ozeki et al., 2004; Ruest et al., 2004; Thomas et al., 1998).

Analysis of the peripheral nervous system at e10.5 by whole-mount immunostaining using anti-NF160 antibody and at e18.5 by histological analysis did not reveal any abnormalities in neural crest cell-specific $G\alpha_q/G\alpha_{11}$ or $G\alpha_{12}/G\alpha_{13}$ mutant mice. No defects of the enteric nervous system, which would result in an aganglionic colon, could be seen when stained with anti-peripherin antibody. Similarly, the adrenal medulla was normally formed and contained tyrosine hydroxylase positive cells, and normal distribution and numbers of melanocytes could also be seen in the skin of e18.5 mutant animals (data not shown).

Mice lacking ET-1 or the ET_A receptor show severe defects in the development of the cardiac outflow tract (Clouthier et al., 1998; Kurihara et al., 1995). In the P0-Cre mouse line, Cre-mediated recombination can be seen in cardiac neural crest cells giving rise to the media of developing arch arteries and the outflow tract septation (Abu-Issa et al., 2002; Hasegawa et al., 2002; Yamauchi et al., 1999) (Figs. 4A–D). At e18.5, P0-Cre-mediated recombination could be seen in the aorta ascendens, the aortic arch, and the ductus arteriosus. However, neither neural crest cell-specific $G\alpha_q/G\alpha_{11}$ nor $G\alpha_{12}/G\alpha_{13}$ mutant animals showed any obvious abnormalities in the cardiac outflow tract when analyzed at e18.5 (Figs. 4E–G). Analysis of the heart, however, revealed severe defects in P0-$Cre^{-/+}$;$G\alpha_{13}^{flox/flox}$;$G\alpha_{12}^{-/-}$ mutant animals, while $G\alpha_q/G\alpha_{11}$ mutants were normal. Animals lacking $G\alpha_{12}/G\alpha_{13}$ in neural crest derivatives consistently had an upper interventricular septum defect and showed an overriding aorta (Figs. 5F–J). In addition, all animals tested had a large cavity within the interventricular septum, which was filled with blood (Figs. 5I,J; Table 1). Serial histological sectioning of hearts showed that this cavity was connected to the left coronary artery (Figs. 5F–J). A vascular origin of the interventricular septum cavity was also suggested by immunohistochemical staining with markers for vascular smooth muscle cells which showed the presence of smooth muscle cells in the wall of the interventricular septum cavity (Figs. 6A,B).

To test for the exact pattern of Cre-mediated recombination in hearts of P0-Cre mice and to study the fate of cardiac neural crest cells in the absence of $G\alpha_{12}/G\alpha_{13}$, we crossed the Cre reporter line Rosa26lacZ to P0-Cre mice as well as to P0-$Cre^{-/+}$;$G\alpha_{13}^{flox/flox}$;$G\alpha_{12}^{-/-}$ animals. The Rosa26lacZ reporter line irreversibly expresses lacZ once cells have undergone Cre-mediated recombination (Soriano, 1999). Staining of the heart for β-galactosidase activity at different stages of development did not reveal any differences between Rosa26lacZ$^{-/+}$;P0-$Cre^{-/+}$ and Rosa26lacZ$^{-/+}$;P0-$Cre^{-/+}$;$G\alpha_{13}^{flox/flox}$;$G\alpha_{12}^{-/-}$ animals (Figs. 6C–E and data not shown)

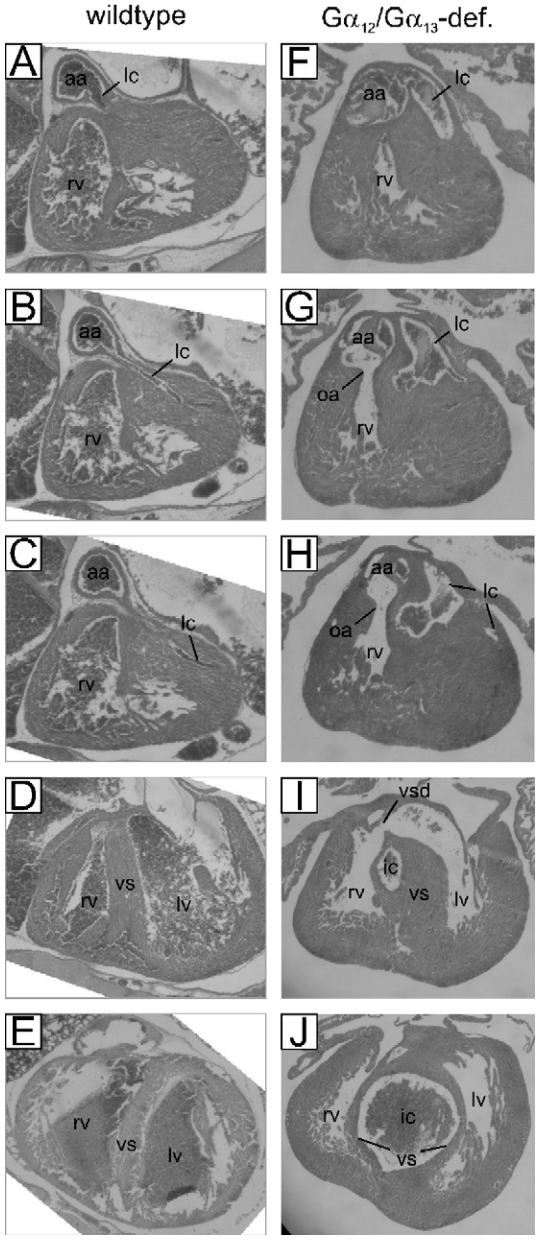

Fig. 5. Heart defects in neural crest cell-specific $G\alpha_{12}/G\alpha_{13}$-deficient mice. Hematoxylin and eosin stained transversal paraffin sections of e18.5 hearts from wildtype (A–E) and $G\alpha_{12}/G\alpha_{13}$-deficient embryos (F–J). An overriding aorta (G,H) and an upper interventricular septum defect (I) as well as an interventricular septum cavity (I,J) connected to the aorta via the left coronary artery (F–I) are consistently present in $G\alpha_{12}/G\alpha_{13}$-deficient embryos. aa—aorta ascendens; rv, lv—right, left ventricle, respectively; lc—left coronary artery; vs—interventricular septum; oa—overriding aorta; vsd—interventricular septum defect; ic—interventricular septum cavity.

indicating that loss of $G\alpha_{12}/G\alpha_{13}$ had no effect on the migration of cardiac neural crest cells. Recombination could be observed in all structures which are believed to derive from the cardiac neural crest including cardiac ganglia, mesenchyme of endocardial cushions, semilunar valves, and the major parts of the aortico-pulmonary and conotruncal septa of the truncus arteriosus (Creazzo et al., 1998; Kirby and Waldo, 1995). However, also some structures like the wall of the

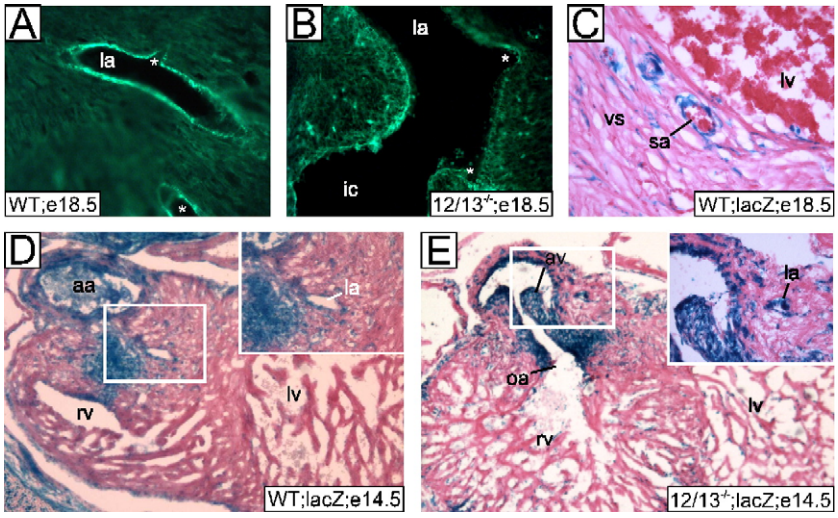

Fig. 6. Composition and origin of the interventricular septum cavity in neural crest cell-specific $G\alpha_{12}/G\alpha_{13}$-deficient mouse hearts. (A,B) Immunofluorescent staining of e18.5 embryonic hearts from wildtype (A) and P0-Cre$^{-/+}$;$G\alpha_{13}^{flox/flox}$;$G\alpha_{12}^{-/-}$ embryos, using an anti-α-smooth muscle actin antibody. (C–E) P0-Cre-mediated lacZ recombination pattern in hearts from e18.5 Rosa26lacZ$^{-/+}$;P0-Cre$^{-/+}$ (C), e14.5 Rosa26lacZ$^{-/+}$;P0-Cre$^{-/+}$ (D), and e14.5 Rosa26lacZ$^{-/+}$;P0-Cre$^{-/+}$;$G\alpha_{13}^{flox/flox}$;$G\alpha_{12}^{-/-}$ embryos (E). Coronary artery formation at e14.5 is not affected in Rosa26lacZ$^{-/+}$;P0-Cre$^{-/+}$;$G\alpha_{13}^{flox/flox}$;$G\alpha_{12}^{-/-}$ mice (inset in E) compared to Rosa26lacZ$^{-/+}$;P0-Cre$^{-/+}$ mice (inset in D). The overriding aorta (oa) is already visible in e14.5 Rosa26lacZ$^{-/+}$;P0-Cre$^{-/+}$;$G\alpha_{13}^{flox/flox}$;$G\alpha_{12}^{-/-}$ embryos (E). la—left coronary artery; aa—aorta ascendens; rv, lv—right and left ventricle, respectively; sa—septal branch of coronary artery; vs—intraventricular septum; av—aortic valve; ic—interventricular septum cavity; *—small coronary arteries.

coronary arteries, which are not of neural crest origin, showed a considerable degree of recombination (Figs. 6C–E).

Discussion

To study the function of G_q/G_{11}- and G_{12}/G_{13}-mediated signaling pathways in neural crest cell development, we generated mice in which the genes encoding $G\alpha_q/G\alpha_{11}$ or $G\alpha_{12}/G\alpha_{13}$ are inactivated in a neural crest cell-specific manner employing the Cre/loxP system. G-protein-coupled receptors for endothelins have been shown to be involved in the development of several neural crest cell-derived structures like the craniofacial tissue, the cardiac outflow tract, enteric ganglia as well as the cutaneous melanocytes (Kedzierski and Yanagisawa, 2001; Kurihara et al., 1999). Other G-protein-coupled receptor systems have so far not been involved in the neural crest cell development. Analysis of mice lacking the ubiquitously expressed G-protein α-subunits $G\alpha_q$ and $G\alpha_{11}$ in all tissues suggests that G_q and G_{11} are involved in the development of cephalic neural crest cells by mediating the effect of the ET-1/ET_A ligand receptor pair (Ivey et al., 2003; Offermanns et al., 1998). Our data show that also neural crest cell-specific deficiency of $G\alpha_q/G\alpha_{11}$ but not of $G\alpha_{12}/G\alpha_{13}$ resulted in craniofacial defects similar to those observed in mice lacking ET-1 or the ET_A receptor (Clouthier et al., 1998; Kurihara et al., 1994; Ruest et al., 2004). The defects in neural crest cell-specific $G\alpha_q/G\alpha_{11}$-deficient mice were more severe than those seen in constitutive $G\alpha_q/G\alpha_{11}$ knock-out animals carrying only one intact $G\alpha_{11}$ allele [$G\alpha_q^{-/-}$;$G\alpha_{11}^{-/+}$] (Offermanns et al., 1998), in which the intact $G\alpha_{11}$ allele may have partially rescued the defect. These data suggest that G_q/G_{11}-mediated signaling in neural crest cells, but not in other cells of the developing craniofacial system is required for its proper development. Defects in the expression of transcription factors like *Dlx3*, *Dlx5*, *eHAND*, and *dHAND*, which are downregulated when endothelin-1/ET_A receptor-mediated signaling is abolished, can be seen in the developing pharyngeal arch mesenchyme at e10.5. In contrast, expression of various factors known to be expressed by migrating neural crest cells in pharyngeal arches was not affected by $G\alpha_q/G\alpha_{11}$ deficiency in neural crest cells consistent with observations made in ET_A receptor- and ET-1-deficient mice. This indicates that the migration of neural crest cells into branchial arches 1 and 2 is not affected by the absence of G_q/G_{11}-mediated signaling in neural crest cells. However, our data support the model that the G_q/G_{11}-mediated signaling system mediates ET_A receptor-dependent differentiation of postmigratory neural crest cells in a cell-autonomous manner. (Clouthier et al., 2000, 2003).

Interestingly, we did not observe any of the other developmental defects of neural crest-derived structures described in mutants of the endothelin system, like defects in the cardiac outflow tract, the enteric ganglia, or the development of melanocytes (Kedzierski and Yanagisawa, 2001). Cre recombination in neural crest cells of the P0-Cre line occurs as early as e9.5 (Abu-Issa et al., 2002; Hasegawa et al., 2002; Yamauchi et al., 1999). The critical period for endothelin-1 and endothelin-3 action on neural crest cells has been reported to be between e9.5 and e10.5 for the development of arterial structures of the outflow tract (Yanagisawa et al., 1998) and e10–e12.5 for proper development of enteric neurons and melanocytes (Shin et al., 1999). Thus, it is unlikely that the lack of additional

characteristic defects in embryos lacking $G\alpha_q/G\alpha_{11}$ and $G\alpha_{12}/G\alpha_{13}$ in neural crest cells and derivatives is due to incomplete recombination. This may indicate that G_q/G_{11} or G_{12}/G_{13} alone do not mediate the effects of the ET-1/ET_A or ET-3/ET_B system on the development of the cardiac outflow tract, enteric nervous system, and melanocytes. Endothelin receptors have also been shown to couple to G-proteins of the G_s- and G_i/G_o-families (Kedzierski and Yanagisawa, 2001; Oksche, 2004) which may mediate some of the developmental effects of the endothelin receptor system. Alternatively, endothelin receptor functions outside the neural crest cell system may contribute to the defects seen in ET-1/ET_A- or ET-3/ET_B-deficient mice.

The perinatal death of neural crest cell-specific $G\alpha_q/G\alpha_{11}$-deficient animals can be explained by mechanical asphyxia due to the malformation of neural crest-derived facial structures. P0-$Cre^{-/+}$;$G\alpha_{13}^{flox/flox}$;$G\alpha_{12}^{-/-}$ mice show a considerable lethality already during the second half of the embryonic period, and no living animals could be recovered postnatally. This is most likely due to cardiac failure resulting from the abnormal development of the septal branch of the left coronary artery, which develops into a huge aneurysma-like structure, which is likely to interfere with the normal pumping function of the heart. It is not clear how this unusual widening of the coronary artery develops. Although there is multiple evidence for the involvement of the cardiac neural crest in the heart development (Creazzo et al., 1998; Farrell et al., 1999), cardiac neural crest cells themselves are unlikely to give rise to the media of coronary arteries (Bergwerff et al., 1998; Poelmann et al., 2002; Waldo et al., 1994), which appears to be of mesodermal origin and to develop from the proepicardial organ (Bernanke and Velkey, 2002; Morabito et al., 2002). Nevertheless, cardiac neural crest-derived cells have been described to surround coronary vessels and they have been suggested to contribute to coronary vessel development (Bogers et al., 1993; Hood and Rosenquist, 1992; Hyer et al., 1999; Waldo et al., 1994).

As we found a considerable degree of recombination in coronary vessels of P0-Cre mice, an alternative explanation could be that coronary vessel malformation in P0-$Cre^{-/+}$; $G\alpha_{13}^{flox/flox}$;$G\alpha_{12}^{-/-}$ mice is the result of $G\alpha_{12}/G\alpha_{13}$ deficiency in the wall of coronary blood vessels. The regulation of vascular smooth muscle cell tone is under the control of the phosphorylation state of the myosin light chain (Somlyo and Somlyo, 1994). Recent evidence indicates that the G_{12}/G_{13}-mediated signaling pathway, which leads to the activation of RhoA and Rho-kinase, is involved in vascular tone regulation. Activation of G_{12}/G_{13} results in Rho/Rho-kinase-mediated inhibition of the myosin phosphatase (Gohla et al., 2000; Somlyo and Somlyo, 2000, 2003). Thus, loss of G_{12}/G_{13}-mediated signaling may result in a reduced vascular tone, which under certain conditions could lead to an abnormal dilatation of the vessel. Future work employing vascular smooth muscle cell-specific inactivation of $G\alpha_{12}/G\alpha_{13}$ genes will be required to test this hypothesis.

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